Research Report

Derivatization of Controlled Pore Glass Beads for Solid Phase Oligonucleotide Synthesis

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ABSTRACT

An improved and simplified procedure for the attachment of nucleosides onto long chain alkylamine controlled pore glass beads (LCAA-CPG) is presented. This procedure uses 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (DEC) to couple nucleoside 3'-succinates directly to the LCAA-CPG. The preparation of nucleoside 3'-succinate anhydrides, p-nitrophenyl, or pentachlorophenyl esters and the use of highly toxic dicyclohexylcarbodiimide (DCC) is no longer required. Procedures involving acidic activation of the LCAA-CPG before derivatization and a pre-synthesis capping are also described, which prevent the formation of oligonucleotides linked by 3'-phosphates to the LCAA-CPG. Evidence is presented indicating that this type of linkage is responsible for the apparently greater than 100% coupling yields observed for the first coupling cycle.

INTRODUCTION

Remarkable improvements in the solid phase synthesis of oligonucleotides has resulted in the proliferation of automated DNA synthesizers (4). These instruments utilize phosphoramidite synthesis chemistry (1,9, 21,22) to assemble sequences on controlled pore glass (CPG) beads (1,10, 13). These CPG beads are preferred because they are uniformly-sized-rigid particles which do not cause clogging or back-pressure problems as did earlier silica gel supports (16).

CPG supports are commercially available with a variety of surface ligands already in place. For oligonucleotide synthesis, the Long Chain Alkylamine (LCAA-CPG) support is used almost exclusively. The LCAA-CPG needs only a terminal nucleoside unit attached to be ready for synthesis. This nucleoside is attached to the support by esterification through a succinic acid molecule to yield surface loadings of approximately 10-40 µmol/g (3,5,10,12,13,20-22).

However, we noticed two problems which appeared to be related to the conventional method of derivatizing the LCAA-CPG (Figure 1, path A). Firstly, the derivatization procedure was lengthy and the nucleoside loadings obtained were quite variable. Secondly, we noticed that conventional derivatization, even with extended capping treatment, could not entirely cap off all of the available sites. The subsequent addition of nucleotide molecules to these unblocked sites during synthesis created the illusion of greater than 100% coupling yields when the trityl colors were monitored.

In this manuscript we describe an alternative derivatization procedure in which the LCAA-CPG is first activated by an acidic pre-treatment. The LCAA-CPG is then coupled to a 2'-deoxyribonucleoside **2a-d** or ribonucleoside-3'-succinate **2e** by the water soluble carbodiimide, 1 - (3 - dimethylaminopropyl) - 3 - ethylcarbodiimide (DEC, sometimes called 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) (6, 8, 11). Finally, underderivatized sites on the support are blocked with acetic anhydride / 4 - dimethylaminopyridine (DMAP) capping reagent (5).

This procedure eliminates the need to separately prepare and isolate activated succinyl esters and prevents unwanted phosphoramidite reaction with the surface of the support. The coupling of the nucleoside 3'-succinates with the support is also much faster and can be completed in as little as 1 h.

MATERIALS AND METHODS

Nucleoside phosphoramidites were prepared by phosphorylation of the appropriately protected nucleoside with N, N - diisopropylmethylphosphoramidic chloride (Aldrich Chemical, Milwaukee, WI) according to previously described methods (3,18,21, 22). Uncoated CPG and LCAA-CPG were from Pierce Chemical (Rockford, IL) in the 500 Å pore and 63-200 µm diameter sizes. Solid phase synthesis was performed on either a custom assembled DNA/RNA synthesizer (18), or on Applied Biosystems 380A or 381A DNA synthesizers using our previously described synthesis cycles (17-19,21,22). DEC was obtained from Sigma Chemical (St. Louis, MO).

Colorimetric Trityl Analysis

Orange trityl washings were diluted with 3% trichloroacetic acid/1,2-dichloroethane solution and the absorbance was measured in one of two

methods. Note: The trichloroacetic acid/dichloroethane solution is preferred over previously used (3,20) perchloric acid solutions for safety reasons. If conversion to molar units was required, the absorbance at 504 nm was measured on a Beckman or Cary spectrophotometer. An extinction coefficient of 76 ml cm^{-1} μmol^{-1} was used to calculate the number of μmol present. If only relative coupling yields were being determined, then measurement was performed using a Brinkmann dipping probe colorimeter equipped with a 470 nm filter and either a 0.4 or a 2 cm probe. This latter procedure reduced the amount of sample dilution required by approximately ten times.

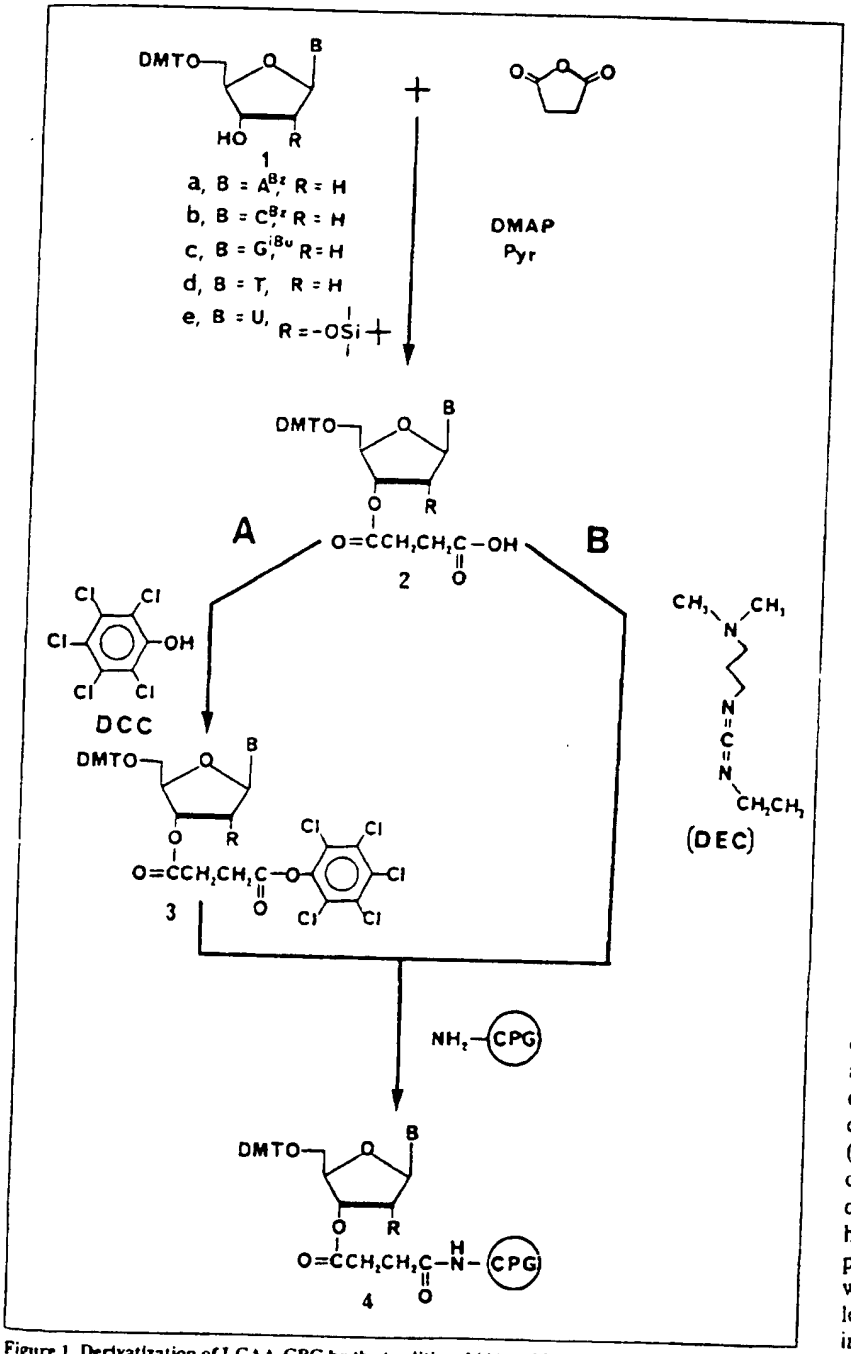

Figure 1. Derivatization of LCAA-CPG by the traditional (A) and improved (B) methods.

Preparation of Nucleoside 3′-Succinates 2a-e

These compounds were prepared using a modified version of the procedure reported by Chow et al. (5). A solution of protected nucleoside **1a-e** (5 mmol), succinic anhydride (15 mmol, 1.51 g), and DMAP (2.5 mmol, 305 mg) was dissolved in anhydrous pyridine (30 ml) and stirred at room temperature (1-3 days) until TLC showed complete reaction. The pyridine was evaporated off and the residue co-evaporated with toluene (3 x 50 ml). The gummy residue was dissolved in $CHCl_3$ and washed with saturated brine solution (3x) and H_2O (1x). The $CHCl_3$ solution was dried with anhydrous Na_2SO_4 and then evaporated to yield a foam (quantitative yield). This material was used without further purification. Note: Compounds **2a-d** have recently become commercially available from Sigma Chemical.

Preparation of Nucleoside 3′-Pentachlorophenylsuccinate Esters 3a-e

Nucleoside 3′-succinate **2a-e** (2 mmol), pentachlorophenol (3 mmol, 799 mg), and DMAP (0.5 mmol, 61 mg) were combined in a round bottomed flask. Melted dicyclohexylcarbodiimide (DCC) (4 mmol, 825 mg) and anhydrous dimethylformamide (20 ml) was then added. *Caution*: Use skin and eye protection when handling DCC. Destroy excess DCC on pipettes, spatulas, etc. by immersion in water, and clean work area with a wet cloth immediately after use to prevent accidental contact. The flask was sealed and left to stir at room temperature (3-4 days). The solvent was removed by coevaporation with anhydrous toluene (3x) to yield a brown gum. This was dissolved in $CHCl_3$ (2-3 ml) and added dropwise to a beaker of rapidly stirred hexanes (250 ml) to precipitate out the product. The precipitation in hexane was repeated until the product was no longer gummy. The product was stored in tightly sealed vials at room temperature.

Pre-activation of LCAA-CPG

LCAA-CPG (10 g) was slowly stirred in a solution of 3% trichloroacetic acid in dichloroethane (200 ml) at room temperature (2-3 h). The LCAA-CPG was filtered off, washed first with $CHCl_3$ and then ether, and dried under vacuum.

Acetic Anhydride/DMAP Capping Reagent

Stock solutions of 0.5 M acetic anhydride in tetrahydrofuran (THF) and 0.5 M DMAP/2,4,6-collidine in THF were prepared separately using THF which had been pre-dried with molecular sieves. After nucleoside derivatization, bulk CPG support may be capped by shaking (1-2 h) in equal parts of the two stock solutions, followed by washing with $CHCl_3$ and drying. Alternatively, the CPG support may be capped on the DNA synthesizer, just prior to synthesis, as described in the text.

Derivatization Method Number 1: Using Nucleoside 3'-Pentachlorophenylsuccinate Derivatives 3a-e

Pre-activated LCAA-CPG (1 g), nucleoside 3'-pentachlorophenylsuccinate **3a-e** (0.25 mmol), and triethylamine (80 µl) in anhydrous pyridine (4 ml) were combined in a sealed 50 ml flask. The mixture was sonicated (1-2 min) to remove trapped gases from the CPG surface and the flask was shaken at room temperature (4-7 days). CPG was filtered off and washed successively with pyridine, $CHCl_3$, and ether.

Derivatization Method Number 2: Using Nucleoside 3'-Succinates 2a-e and DEC

Pre-activated LCAA-CPG (1 g), DMAP (0.1 mmol, 12 mg), nucleoside succinate **2a-e** (0.2 mmol), triethylamine (80 µl), DEC (2 mmol, 382 mg) and anhydrous pyridine (10 ml) were combined in a sealed 50 ml flask. The mixture was sonicated (1-2 min) and then shaken at room temperature for 1-24 h, depending on the loading desired. After shaking, the CPG was filtered off and washed successively with pyridine, methanol, and $CHCl_3$ and then dried under vacuum.

RESULTS AND DISCUSSION

Improved Nucleoside Attachment

Unlike earlier silica gel supports (5), nucleoside succinates cannot be adequately attached to LCAA-CPG beads using DCC. Attempts to do so result in unacceptably low loadings (< 2 µmol/g) even when prolonged reactions are performed. Consequently, it is necessary to activate the nucleoside 3'-succinate to achieve coupling with the amino group on the LCAA-CPG. This may be done by converting the nucleoside 3'-succinate into the symmetrical anhydride (20) or esterifying it with *p*-nitrophenol (3,13) or pentachlorophenol (10,12). All three methods are quite similar since the ester or anhydride formation is brought about by the use of DCC.

In our laboratory we primarily used pentachlorophenol activation to derivatize LCAA-CPG supports (Figure 1, path A) to nucleoside loadings of between 10 and 40 µmol/g. However, the procedure had several disadvantages.

Firstly, synthesis of the activated pentachlorophenylsuccinates required use of the highly toxic materials DCC and pentachlorophenol. Secondly, the preparation of compounds **3a-e** was tedious and gave only moderate yields (50-75%), since multiple precipitations from hexane were required to produce dry powders which were suitable for long-term storage. Thirdly, the coupling reactions were quite lengthy, with 3-4 days required to prepare compounds **3a-e** and an additional 4-7 days required for the coupling to the CPG. Fourthly, the entire procedure had to be repeated every time we wanted to experiment with a different ribonucleoside, modified base, or nucleoside protecting group. Finally, CPG loadings obtained were quite variable, and supports with optimum loading of 30-40 µmol/g were not always obtained.

We sought to improve the procedure by finding a way to couple the nucleoside 3'-succinates **2a-e** directly to the support so that the difficulties involved with the activated succinate ester preparations could be eliminated. We suspected that simple DCC-mediated esterification of **2a-e** to the LCAA-CPG was less than satisfactory, because the large DCC molecule was too sterically constrained near the surface of the CPG. We therefore substituted a smaller and less rigid carbodiimide, DEC, which has been widely used in aqueous formation of phosphate esters (6,8). DEC has also been used to couple peptides to the surface of silica gel supports (11).

When this reagent was shaken with a mixture of **2a-e**, LCAA-CPG, and a catalytic amount of DMAP in triethylamine/pyridine, coupling of the nucleoside to the support was easily obtained (Figure 1, path B). The results obtained for the four common deoxyribonucleosides **1a-d** and the more hindered ribonucleoside **1e** are shown in Table 1. These reactions were initially performed for six days, but later experiments showed that the reactions were complete after only 24 h. The loadings obtained after a 24 h reaction (50-65 µmol/g) were much higher than could be obtained using compounds **3a-e** and the former procedure. These higher nucleoside loadings were quite useful for the synthesis of large amounts of oligonucleotide, since 2 and 20 µmol scale syntheses could be performed, respectively, in columns designed for 1 and 10 µmol scale synthesis.

We have also been able to prepare supports with nucleoside loadings within the conventional range (20-30 µmol/g) by simply decreasing the coupling time to only 1 h. These lower loading supports are preferred for syntheses in which the length of product is more important than the quantity.

Improved Capping Procedure

The second difficulty we experienced with conventionally derivatized LCAA-CPG was the observation of apparently greater than 100% coupling yields when trityl colors from oligonucleotide syntheses were monitored. This anomaly was greatest for the first coupling step, with apparent coupling yields ranging from 120-250%, but could also occur, to a lesser extent, in the second and third couplings. This phenomenon has previously been ascribed to loss of the trityl

Table 1. Nucleoside Loadings Obtained by Coupling 2a-e to LCAA-CPG Using DEC

Nucleoside used	Loading obtained 1 h rxn	24 h rxn	6 day rxn
2a	33 µmol/g	64 µmol/g	65 µmol/g
2b	28	64	55
2c	23	51	43
2d	30	65	55
2e	10	67	66

Table 2. Effect of Pre-Capping on the First Coupling Yield and on the Amount of Material Which Cannot Be Cleaved from the Support. Synthesis of dDMT-TTC Using a 1 µmol Scale Cycle and Acid Pre-Activated and Derivatized LCAA-CPG

Pre-Capping time[a] (min)	Loading after first coupling (µmol/g)	Yield (%)[b]	Material remaining after Deprotection[c] (µmol/g)	(%)[b]
0	75.6	270%	29.8	106%
1	33.3	119%	4.7	16%
2	30.2	108%	2.5	9%
5	30.2	108%	2.5	9%
10	27.7	99%	1.5	5%
30	28.3	101%	1.2	4%
60	27.7	99%	1.5	5%

[a]Treatment with acetic anhydride/DMAP capping reagent prior to synthesis

[b]Based on an original CPG loading of 28 µmol/g of **2b**

[c]Automatic synthesizer deprotection cycle consisting of: (1) Thiophenol/triethylamine/dioxane 1:1:2 (30 min) and (2) 15 M NH_4OH, room temperature (6 x 15 min)

group from the first nucleoside on the CPG, by spontaneous detritylation during storage (2). However, we believed that this explanation was incorrect based on the following observations.

Firstly, we have stored, at room temperature, derivatized LCAA-CPG supports in our laboratory for over four years without the loss of either trityl or nucleoside groups. The absence of detritylation during storage was confirmed by comparing results determined from trityl analysis with results obtained by nucleoside hydrolysis.

Secondly, we found that treatment of the support with powerful capping reagents, such as diethylphosphorochloridite/triazole (16), methyl dichlorophosphite (16), diethyl-N,N-diisopropylphosphoramidite/tetrazole (18), or acetic anhydride/DMAP (5) before synthesis did not reduce the apparently greater than 100% coupling yields.

Thirdly, when the trityl colors were quantitated, the oligonucleotide loadings on the CPG increased by approximately 150-200%, from 20-40 µmol/g to 40-60 µmol/g. The exact loadings in each case were determined by colorimetric analysis. These large increases could not be attributed to detritylation of the original supports, since the observed loadings were even higher than the amount of primary amine group (30-40 µmol/g) reported to be on the surface of CPG by Pierce Chemical Co.

Fourthly, when LCAA-CPG supports were examined after the automatic deprotection/cleavage cycles of a number of Tr-ON/auto (5'-terminal trityl group left on) oligonucleotide syntheses, significant amounts of tritylated oligonucleotide were found remaining on the support (*cf.* Table 2). This material was resistant to extended hydrolysis with ammonium hydroxide but could be detected by washing the "deprotected" CPG with trichloroacetic acid solution and quantitating the released trityl cations.

The above observations led us to hypothesize that the additional trityl color detected arose from reaction of the activated phosphoramidite reagents with sites on the surface of the support. This would necessarily result in oligonucleotide sequences coupled to the CPG via a 3'-phosphate ester or amide linkage. Such linkages would be converted into phosphodiester or phosphoramidate linkages during the phosphate deprotection step and would be highly resistant to subsequent hydrolysis. This resistance would prevent cleavage from the support and explains why such sequences have not been detected until now.

The failure of the capping reagents to block off the reactive sites on the surface could be accounted for by assuming that the reactive sites on the surface of the support were not present until activated or unmasked by the first synthesis cycle. In particular, we suspected that the acidic conditions of the detritylation steps might be affecting the surface of the support.

To investigate these hypotheses, we took underivatized LCAA-CPG support (i.e. no nucleoside attached), washed it for various times with 3% trichloroacetic acid/dichloroethane solution, loaded it into a synthesis column, and performed a synthesis cycle on it using 5'-dimethoxytritylthymidine - 3' - diisopropylmethyl phosphoramidite. The amount of phosphoramidite reaction with the support was determined by trityl analysis and the results are shown in Table 3. These experiments confirmed our hypotheses by proving that (1) nucleotides could be attached to the LCAA-CPG support via 3'-phosphate ester or amide linkages (no other linkages were possible), and (2) that the number of reactive sites on the surface increased when treated with the acidic detritylation reagent. Control experiments using either uncoated CPG or underivatized Vydac silica gel showed that the effect of the trichloroacetic acid reagent was limited to LCAA-CPG.

As expected, the phosphate linkage to LCAA-CPG was resistant to standard deprotection conditions (thiophenol/triethylamine/dioxane 1:2:2, 30 min, followed by 28-30% ammonium hydroxide, 90 min). UV quantitation of

Table 3. Phosphoramidite Reaction[a] with Acid Pre-Activated Underivatized LCAA-CPG

Acidic[b] Pre-Activation	Amount of DMT-Tp Attached to the Surface of Support
0.0 min	6 µmol/g
1.5 min	23 µmol/g
2 h	71 µmol/g
24 h	80-85 µmol/g

[a]Synthesis was performed using a standard 1 µmol synthesis cycle

[b]3% Trichloroacetic acid/ 1,2-dichloroethane

the material released after room temperature hydrolysis with NH_4OH showed that only 20-30% of the bound thymidine was released. Reversed-phase HPLC analysis indicated that this recovered material was mostly thymidine-3'-methylphosphate along with just a small amount of thymidine-3'-phosphate.

The maximum loading (80-85 µmol/g) obtained during these experiments was approximately double the amino loading of the support (40 µmol/g). This suggested the possibility of another masked amino or hydroxyl group on the support, which would only be accessible after acidic deblocking. A structure for the LCAA ligand has been reported (20) which includes a second hexamethylene diamine group on the ligand (Figure 2), but it does not specify the presence or absence of any protecting groups. This structure could account for our results, but we have not been able to confirm our hypothesis with the Pierce Chemical Company since the exact side chain structure remains proprietary.

The solution to the anomalous first coupling yields was therefore to pre-activate the support with an acidic wash *before* the derivatization with the desired nucleoside. After the attachment of the first nucleoside, the amine or hydroxyl groups remaining were then blocked off by treatment with acetic anhydride/DMAP.

The time required to completely acetylate the remaining groups was determined by treating portions of acid preactivated derivatized support with capping reagent for various intervals just prior to synthesis of a trinucleotide. The coupling yield of the first base addition was determined by measurement of the released trityl colors (Table 2). A second base addition was also performed, and this time the 5'-terminal trityl protecting group was not removed. The automatic deprotection cycle was used to remove the methyl protecting groups and cleave the sequences from the support. Finally, deprotected supports were washed with acetonitrile and dried under vacuum. The retained trityl loadings were then determined by treating the deprotected support with trichloroacetic acid solution and quantitating released trityl color (Table 2).

These experiments showed that a pre-capping step of between 10-30 min was sufficient to block off the residual amine groups. Without a capping step of this duration, the residual amine or hydroxyl groups created apparent coupling yields of between 108% and 270% by reacting with the phosphoramidite reagent. Depending on the amount of excess coupling, retained trityl loadings of from 2-30 µmol/g were detected on the supports, after they had been deprotected. This indicated the amount of material attached to the support via 3'-phosphate ester or amide linkages.

We have also tried bulk capping of the CPG support immediately after the nucleoside derivatization step. However, material prepared in this manner slowly loses the acetyl blocking group and needs to be re-capped after 2-3 months storage. To avoid this problem, we prefer to cap the support just prior to the start of synthesis. This may be easily performed on the DNA synthesizer by use of an automatic or manual capping cycle.

The importance of completely eliminating reaction of the phosphoramidites with the surface of the support varies with the type of synthesis being performed. Small-scale syntheses, which will be used in biological studies, are probably not affected by this side reaction, since most of the excess material remains attached to the CPG and is discarded. The remaining trace amounts are either removed during purification or, due to their 3'-phosphorylated ends, have no effect. However, larger-scale syntheses, which use a lower excess of phosphoramidite, can be affected since the unwanted side-products are competing for the incoming reagents. In either case, this side reaction can distort the overall efficiency of the synthesizer, and the greater than 100% first coupling yields should never be used in the calculation of average or overall coupling yields (2).

The modified derivatization procedures described above have been in use in our laboratories for over three years, and almost 1500 DNA or RNA (21-23) sequences, including a number of large-scale (30-50 mg product) syntheses (7,14, 15), have been prepared.

In summary, the improved nucleoside coupling procedure described in this manuscript greatly reduces the amount of effort and time required to prepare LCAA-CPG supports. Trityl based coupling yields of greater than 100%, caused by formation of 3'-phosphate ester or amide links to the support, are also eliminated. Furthermore, experience with synthetic manipulations is no longer required, since the difficult preparation of intermediates 3a-e is eliminated. Instead, all of the required compounds can be commercially obtained, and the derivatization procedure is only a matter of combining the materials together and shaking them. After coupling, the derivatized

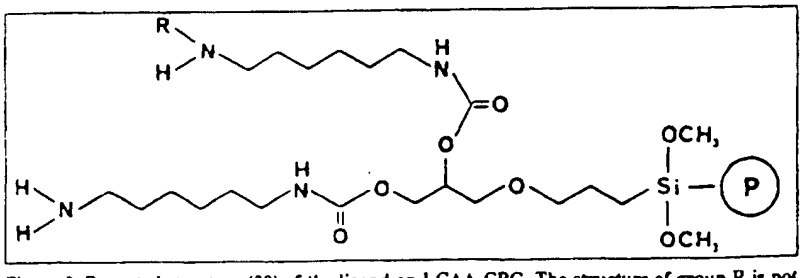

Figure 2. Reported structure (20) of the ligand on LCAA-CPG. The structure of group R is not known.

support needs only to be filtered off, washed and dried.

The preparation of these supports within the laboratory can also significantly reduce costs. For example, a typical 5 g CPG preparation requires only about $150 worth of materials but will yield sufficient material for 500 0.2 µmol scale syntheses. The simplicity and economy of this method should therefore increase the number of laboratories which can prepare these important yet expensive materials.

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